I. REMARKS

Claims 1 to 18 and 27 to 33 are pending. Claims 4, 5, 17, 18, and 29 have been withdrawn.

It is noted that, while the Office Action Summary indicates that claims 11 to 18 and 27 to33 are pending, the Examiner confirmed by telephone that claims 1 to 18 and 27 to 33 are pending.

A. Regarding the Amendment

Claims 1 and 27 have been amended to clarify that an antisense polynucleotide useful for practicing the claimed methods "inhibits hexokinase gene expression". The amendments are supported, for example, at page 5, lines 1-14, and, therefore, do not add new matter.

B Regarding the Restriction Requirement

The withdrawal of claim 29 and the recognition of claims 27, 28 and 30-33 are generic linking claims is acknowledged. As such, it is requested that upon determining that the linking claims are allowable, the restriction requirement be withdrawn with respect to the linked inventions, and any claims depending from or otherwise including all the limitations of the allowable linking claims be entitled to examination in the instant application. Claims withdrawn pursuant to the restriction requirement remain pending.

C. Rejections under 35 U.S.C. § 112

The rejection of claims 1 to 3, 6 to 16, 27, 28, and 30 to 33 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite is respectfully traversed.

It is stated in the Office Action that the claims are indefinite as to the conditions required for hybridization of the oligonucleotide with a mRNA encoding a hexokinase. Applicants point out that claims 1 and 27 have been amended to delete reference to "an oligonucleotide that

hybridizes" with a mRNA encoding a hexokinase, thus clarifying that an antisense hexokinase polynucleotide, which inhibits hexokinase gene expression, is used to practice the claimed methods. Further, it is noted that the claims require "contacting the cells" with the antisense polynucleotide. As such, it is submitted that in view of the claims, and of the well known functional characteristics of antisense polynucleotide (see, e.g., page 5, lines 1-14), one skilled in the art would recognize that the hybridization between an antisense polynucleotide and an mRNA encoding a hexokinase is such that it occurs under the physiological conditions present in the cells being contacted. Accordingly, it is submitted that one skilled in the art, viewing the claims, would clearly know the metes and bounds of the claimed subject matter and, therefore, respectfully requested that the rejection of the claims under 35 U.S.C. §112, second paragraph, be removed.

The objection to the specification and corresponding rejection of claims 1 to 3, 6 to 16, 27, 28, and 30 to 33 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement are respectfully traversed.

The Examiner has maintained the rejections for the reasons set forth in the previous Office Action (Paper No. 17), and further clarifies that the claimed subject matter lacks enablement because it would be unclear, in light of Newgard et. al., how one would inhibit only tumor cells and not inhibit other cells. As an initial matter, Applicants point out that the Examiner appears to be reading limitations into the claims that are not, in fact, recited. Specifically, while the claims require "inhibiting the proliferation of tumor cells characterized by having a highly glycolytic phenotype", the claims do not address any effect that may occur on non-tumor cells and, specifically, do not require that the antisense polynucleotide not inhibit proliferation of normal cells. In this respect, Applicants point out that many agents used to treat cancers, including, for example, chemotherapeutic agents, are not limited in their effect to cancer cells, but also have a deleterious effect on normal cells. Nevertheless, such agents are useful, and

commonly used, for treating cancer. As such, Applicants submit that the fact that the methods of the invention may result in inhibiting proliferation of cells other than tumor cells in a subject does not impact the usefulness of the methods, and is not at all relevant to enablement of the claimed methods.

Further with respect to enablement, Applicants point out that the specification discloses that an antisense hexokinase molecule reduced hexokinase activity by as much as 93% in tumor cells having a highly glycolytic phenotype due to hexokinase activity, and that decreased cell proliferation correlated with the decreased hexokinase activity (see page 40, lines 20-26; page 41, lines 1-3; and Figures 4 and 5). Furthermore, the specification discloses that expression of hexokinases occurs in various tissues and that a highly glycolytic phenotype due to hexokinase activity has been identified in various types of tumor cells (see, e.g., page 9, line 5 to page 10, line 14). Thus, the specification discloses that elevated hexokinase is associated with a highly glycolytic phenotype in tumor cells, and exemplifies the use of an antisense Type II hexokinase molecule to inhibit the proliferation of such tumor cells. As such, and as previously set forth in Applicants' response mailed October, 20, 2003, one skilled in the art would not have had any reason to believe that that the claimed methods would not be equally effective in reducing the proliferation of highly glycolytic tumor cells other than hepatoma cells. As such, it is submitted that one skilled in the art, viewing the specification, reasonably would have known that the claimed methods can inhibit the proliferation of any tumor cells exhibiting a highly glycolytic phenotype.

It is further alleged that the claims lack enablement because the specification does not demonstrate inhibition of tumor cell proliferation due to inhibition of only a Type I hexokinase or only a Type II hexokinase. As discussed above, however, it again appears that the rejection is based on limitations that are not present in the claims. The claims are not directed, for example, to a method of inhibiting Type II, but not Type I, hexokinase gene expression, but, instead, are

directed to a method of inhibiting the proliferation of tumor cells having a highly glycolytic phenotype, by contacting the tumor cells with an antisense polynucleotide that hybridizes to a hexokinase mRNA (claim 1) or to a Type I or Type II hexokinase mRNA (claim 27) and inhibits hexokinase gene expression, thereby inhibiting proliferation of the tumor cells. As such, it is submitted that the mechanism by which the invention works (i.e., whether Type I hexokinase, or Type II hexokinase, or both Type I and Type II hexokinase are inhibited) is not relevant to enablement of the claimed invention (see, also, *Cross v. Iizuka* 224 U.S.P.Q 739 (Fed. Cir. 1985); footnote 3).

The claims are directed to methods of inhibiting proliferation of tumor cells having a highly glycolytic phenotype by contacting the cells with an antisense polynucleotide that hybridizes with a hexokinase mRNA (claim 1) or a Type I hexokinase mRNA or Type II hexokinase mRNA and inhibits hexokinase gene expression. The specification exemplifies an antisense hexokinase polynucleotide comprising Type II hexokinase cDNA in an antisense orientation (page 38, line 20, to page 39, line 5), and further discloses that, due to the close homology of the Type I and Type II hexokinase isotypes, it is highly likely that the exemplified antisense polynucleotide would hybridize with a Type I hexokinase mRNA or a Type II hexokinase mRNA (see, e.g., page 11, line 13, to page 12, line 11; and paragraph bridging pages 40 to 41; see also, Applicants' response mailed May 12, 2003, at page 7, first paragraph). Furthermore, as discussed above, the specification discloses that the claimed method, in fact, inhibits proliferation of tumor cells characterized by a highly glycolytic phenotype (see page 40, lines 20-26). As such, regardless of the mechanism by which the claimed methods work, the skilled artisan, viewing the subject application, would have known that contacting tumor cells characterized by a highly glycolytic phenotype with an antisense hexokinase polynucleotide inhibits proliferation of the tumor cells.

In summary, the specification discloses that proliferation of tumor cells having a highly glycolytic phenotype can be inhibited by contacting the cells with an antisense hexokinase

polynucleotide, and further discloses that various types of tumor cells are characterized by a highly glycolytic phenotype associated with elevated hexokinase activity. As such, and absent objective evidence to the contrary, it is submitted that the skilled artisan, viewing the subject application, would have known how to practice the claimed methods without undue experimentation.. Accordingly, it is respectfully requested that the Examiner reconsider and remove the rejection of the claims as allegedly lacking enablement under 35 U.S.C. 112, first paragraph.

The rejection of 1 to 3, 6 to 16, 27, 28, and 30 to 33 under 35 U.S.C. 112, first paragraph, as allegedly lacking written description is respectfully traversed.

It is acknowledged in the Office Action that the specification adequately describes the antisense nucleic acid sequence set forth as SEQ ID NO:1. It is alleged, however, that the specification does not describe the structure of possible antisense targets or any other antisense molecule other than the full expressed SEQ ID NO:1 in antisense orientation. It is further stated that the exemplified antisense molecule inhibits both Type I and Type II hexokinase, "while the scope of the instant invention embraces the inhibition of only a Type II hexokinase." (see Office Action, page 13, first paragraph). As discussed above, however, the Examiner appears to read this limitation into the claim; however, the claims are not directed to "inhibition of only a Type II hexokinase". As such, it is requested that this basis of the rejection be removed for the reasons set forth above.

With respect to the description of antisense molecules useful for practicing the claimed methods, Applicants point out that hexokinases have been characterized in healthy and cancerous tissues, including mapping of genetic structure (page 10, lines 15-30, and page 11, lines 13-32), and the specification discloses full length cDNA sequences of Type I (SEQ ID NO:2) and Type II (SEQ ID NO:1) hexokinase. In addition, the specification discloses an antisense molecule corresponding to SEQ ID NO:1, which is representative of antisense polynucleotides useful for

practicing the claimed methods, and discloses that antisense polynucleotides useful for the present methods can include a sequence complementary to a portion of the target molecule (e.g., polynucleotides that are 100-3000 nucleotides in length; page 13, lines 20-21). Further, the specification discloses that antisense polynucleotide must be capable of forming a stable duplex with a portion of the mRNA target such that the specific hybridization interferes with the normal function of the nucleic acid, and provides methods of determining stable duplex formation (see, e.g., page 20, line 31, to page 21, line 12; and, page 22, lines 2-25), as well as guidance in determining proper polynucleotide length (page 20, lines 22-30).

Further in this respect, Applicants point out that methods for designing antisense molecules based on knowledge of the target RNA were well known at the time the subject application was filed (see, e.g., Exhibit A, which was submitted for publication before the priority date of the subject application, and refers to earlier references citing similar methods; see, e.g., page 2603, right column, citing to refs. 3 (1998) and 6 (1999)). In addition, commercial services for preparing antisense molecules based on target mRNA information are known in the art (see, e.g., Exhibit B, "order form" from MoleculA (Sterling VA) website).

Although it is acknowledged in the Office Action that the specification discloses methods of identifying antisense molecules, it is alleged that such methods, without providing the polynucleotides themselves, is not sufficient to meet the written description requirement. However, as set forth in MPEP § 2163 II.A.3.(a):

> what is conventional or well known to one of ordinary skill in the art need not be disclosed in detail...If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.

Applicants submit that, in the present case, in which the specification discloses an effective antisense molecule, as well as the target hexokinase sequences and methods of identifying antisense molecules, and further in view of Exhibits A and B submitted herewith, that one skilled

in the art would have known that Applicants were in possession of the full scope of antisense molecules useful for practicing the claimed methods.

It is also stated in the Office Action the antisense polynucleotide of claims 2 and 28 have been interpreted to not be limited to molecules fully complementary to SEQ ID NO:1, and, therefore, can encompasses two nucleotides of SEQ ID NO:1 but further complementary to some other hexokinase. Applicants point out, however, that the claims have been amended to delete reference to "oligonucleotides" and, therefore, encompass only "antisense" molecules. In this respect, Applicants point out that antisense polynucleotides, in addition to be complementary to a target molecule, further have a functional aspect in that they inhibit expression of the target gene product (i.e., inhibit translation of hexokinase from the target hexokinase mRNA) due to specific binding and formation of a stable duplex with the target molecule. Applicants submit that one skilled in the art would not reasonably consider a polynucleotide containing two nucleotides to SEQ ID NO:1 to be an antisense polynucleotide specific for a hexokinase, including a Type I hexokinase and/or a Type II hexokinase. As such, in view of the amendment to the claims, it is respectfully requested that this basis of the rejection be withdrawn..

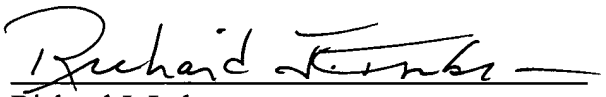
For the reasons set forth above, it is submitted that the specification provides adequate disclosure and guidance such that one skilled in the art would have recognized that Applicants were in possession of the claimed methods. Accordingly, it is respectfully requested that the rejection of the claims as allegedly lacking a written description requirements under 35 U.S.C. §112, first paragraph, be removed.

In view of the amendments and the above remarks, it is submitted that the claims are in condition for allowance, and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicants' undersigned representative if there are any questions relating to this application.

No fee is deemed necessary in connection with the filing of this communication. However, if a fee is required, the Commissioner is hereby authorized to charge any required fee associated with this communication, or credit any overpayments, to Deposit Account No. 50-1355.

Respectfully submitted,

Date: June 22, 2004



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Theoretical design of antisense genes with statistically increased efficacy

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ABSTRACT

Endogenous expression of antisense RNA represents one major way of applying antisense nucleic acids. To express antisense RNA intracellularly, recombinant antisense genes have to be designed and introduced into cells where the target RNA is encountered. Efficient annealing between the antisense RNA and the target RNA is crucial for efficacy and is strongly influenced by RNA structure. Here we extend structural rules for the design of *in vitro* transcribed antisense RNAs to the design of recombinant antisense genes. Intracellularly expressed antisense RNA transcripts contain a central antisense portion and additional flanking vector-derived sequences. A computer algorithm was generated to compose large sets of antisense genes, to calculate secondary structures of the transcribed sequences and to select for favorable structures of antisense RNA in terms of annealing and efficacy. The biological test system to measure efficiency of antisense genes was human immunodeficiency virus type 1 (HIV-1) replication in 293T cells. When considering the lower intracellular steady-state levels of favorably structured endogenous transcripts, an antisense effect against HIV-1 replication was observed that was up to 60-fold stronger than that measured for predicted unfavorable species. The computational selection was successful for antisense portions of 300 nt but not 100 nt in length. This theoretical design of antisense genes supports their improved application under time- and labor-saving conditions.

INTRODUCTION

Accumulating evidence suggests that the efficacy of long chain antisense RNA in living cells is related to fast annealing of the antisense RNA and the target RNA *in vitro* and, presumably, also *in vivo* (1,2). Recent insights into the relationship between structural elements and annealing kinetics of artificial antisense RNAs indicate that global flexibility and a high number of external nucleotides of *in vitro* transcribed HIV-1 *gag*-directed antisense RNA without additional non-complementary sequences favor both RNA–RNA annealing *in vitro* and efficacy in living cells (3). Recombinant antisense genes, rather than *in vitro* transcribed antisense RNA, represent one of the major forms for the application of antisense inhibitors. The relationship between RNA structure and efficacy in this case is complicated by stretches of vector-derived transcribed sequences that usually flank the central antisense portion of endogenously expressed antisense RNA. As a major consequence it seems to be extremely difficult to establish experimental procedures to identify effective antisense genes out of the space of all possible antisense genes against a given target at sufficient reliability and at appropriate expense. Conversely, computational approaches may serve as an alternative tool to select effective antisense genes provided that parameters can be defined that are accessible to computer calculation and that are related to the efficacy of antisense genes in living cells.

To test this possibility, we developed an algorithm that generates all possible endogenous antisense transcripts against a given target sequence. Target sequences and their structures are usually defined by the biological system of interest and the chosen target system whereas antisense species can be selected out of the complete antisense sequence space. To minimize the influence of the target sequence on the efficacy of antisense nucleic acids tested here, we chose HIV-1 *gag* sequences for several reasons. First, *gag* target sequences are relatively homogeneous with respect to the local folding potential (4), i.e. within the RNA encoding gag, the occurrence of extremely stable or unstable sub-structures is unlikely. This is especially true for target regions longer than 100 nt. Secondly, *gag*-directed antisense RNA has already been shown to efficiently inhibit HIV-1 replication in human cells (5). Thirdly, despite extensive splicing within transcripts of HIV-1, the *gag* target sequence used here occurs only on the unspliced RNA *in vivo*, i.e. the target RNA in infected living cells is defined.

Here, transcribed plasmid-derived sequences and all possible consecutive 100 or 300 nt long antisense sequences directed against HIV-1 *gag* were combined by computer and the secondary structures of all antisense transcripts were calculated and recorded. The selection criteria for favorable antisense transcripts were, in principle, those that had recently been shown to be relevant (3,6). The highest ranking was assigned to structures with the highest global flexibility, reflected in high numbers of external unpaired nucleotides and

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structural components. To average over individual properties, we cloned a total of 12 favorably predicted HIV-1 *gag*-directed antisense genes and seven antisense constructs predicted to behave less effectively. To compare the anti-HIV-1 efficacy of different antisense genes, we considered intracellular RNA steady-state levels of antisense transcripts. These were used to calculate relative antisense effects. The results indicate that the length of the antisense stretch is a critical parameter. The computational design described in this work can significantly and reliably improve the efficiency of antisense genes.

MATERIALS AND METHODS

Computer-aided design

The computational selection was perfomed using a modification of the algorithm Foldanalyze (3), which is based on the program Mfold v.2.0 (7) and which is included in the Heidelberg UNIX Sequence Analysis Resources (HUSAR) (8). The program Space was developed to characterize the antisense structure spaces.

Construction and preparation of plasmid DNA

For construction of plasmids harboring antisense genes we used plasmid pEGFP-C1 (Clontech, Palo Alto, CA). The gfp coding sequence was deleted between the *Eco*47III and *Sma*I sites. The selected PCR fragments from HIV-1 proviral DNA clone pNL4-3 (EMBL accession no. REHIVNL4) were subcloned into the *Mlu*I and *Nde*I restriction sites of the polylinker. Plasmid DNA was amplified in *Escherichia coli* strain XL1-blue, isolated and purified by cesium chloride gradient centrifugation. The purity was monitored by agarose gel electrophoresis (1% agarose). The DNA concentration was measured by UV absorption at 260 nm.

Cell lines and cell culture

Cell line 293T was cultured in DMEM medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C and passaged twice a week. For transfection experiments the cells were seeded in 48-well culture plates at a density of 2×10^4 cells/well and grown to half confluence (12 h in culture).

Transfection and inhibition of HIV-1 replication

HIV-1 replication was measured 24 h after calcium phosphate co-transfection (9) of 150 ng HIV-1 proviral DNA (clone pNL4-3) and 250 ng plasmid DNA per well. HIV-1-specific antigen concentrations in the culture supernatants were determined by ELISA (Organon Teknika, Boxtel, The Netherlands). Values were standardized to the effect of the cloning vector (100% p24 expression).

Determination of intracellular RNA steady-state levels

293T cells were transfected as described above without HIV-1 proviral DNA and incubated for 24 h at 37°C. The cells were removed from the culture plates by trypsinization and RNA was isolated using the RNeasy protocol (Qiagen, Hilden, Germany). Isolated RNA was reverse transcribed (Reverse Transcription Reagents; PE Biosystems, Foster City, CA) and amplified by quantitative PCR (SYBR Green PCR Core Reagents, Gene Amp Sequence Detector 5700; PE Biosystems) using forward primer 5′-GATCCGCTAGCGGGATCC-3′ and reverse primer 5′-CCTCTACAAATGTGGTATGGCTGA-3′ for the HIV-1 *gag*-directed antisense RNA and forward primer 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse primer 5′-GAAGATGGTGATGGGATTTC-3′ for *GAPDH*, which was used as an internal standard.

RESULTS

Theoretical basis of the design of antisense genes

Recent observations on the relationship between predicted secondary structures of 100 nt long antisense RNA and their efficacy in mammalian cells suggested that specific structural features are related to effectiveness (3). First, high numbers of external bases, i.e. nucleotides that are not involved in base pairing and that do not belong to structural elements. Thus external bases include free ends and joint sequences but not unpaired nucleotides within loops and bulges. Secondly, high numbers of structural components, i.e. structural folding units that are linked via flexible joint sequences to each other. These observations cannot be simply transferred to endogenously expressed fusion transcripts with 5′- and 3′-terminal non-antisense sequences which are likely to affect folding of the central antisense sequence.

For the computational design of antisense genes the following algorithm was used: (i) a given target sequence of L nucleotides in length is used to generate the complete relevant antisense sequence space [complexity = $L/2 \times (L + 1)$]; (ii) constant vector-encoded sequences that are co-transcribed within the antisense sequence-containing expression cassette, such as promotor element-derived sequences or termination signals, are attached to the 5′- and 3′-end, respectively; (iii) the five lowest free energy secondary structures are predicted for all species of the resulting virtual space of antisense transcripts; (iv) all resulting secondary structures which are conserved among the five lowest free energy foldings are ranked according to given selection rules for efficiently annealing structures such as those described above. Highest ranking was given to structures with primarily the highest numbers of external bases and additional high numbers of components. No cut-off was used.

Computational selection of HIV-1 *gag*-directed antisense RNA structures

The parental expression plasmid used in this work was plasmid pEGFP-C1. Its gfp coding sequences were replaced by the selected antisense sequences. In the resulting antisense expression cassettes central antisense sequences were flanked by terminal plasmid-derived constant sequences, 33 nt of the CMV promotor at the 5′-end and 177 nt of the SV40 polyadenylation signal [without the poly(A) tail] at the 3′-end. In order to eliminate possible effects of the length of antisense transcripts on their efficiency in living cells and to be able to analyze a variety of constructs in a comparable way we restricted the computational analysis to molecules containing either a 100 or 300 nt antisense stretch which is coincident with restriction of the analyzed sequence spaces to a diversity of $(L - 100)$ or $(L - 300)$ different molecules respectively. The distributions of external bases and components and their variances along the HIV-1 *gag*

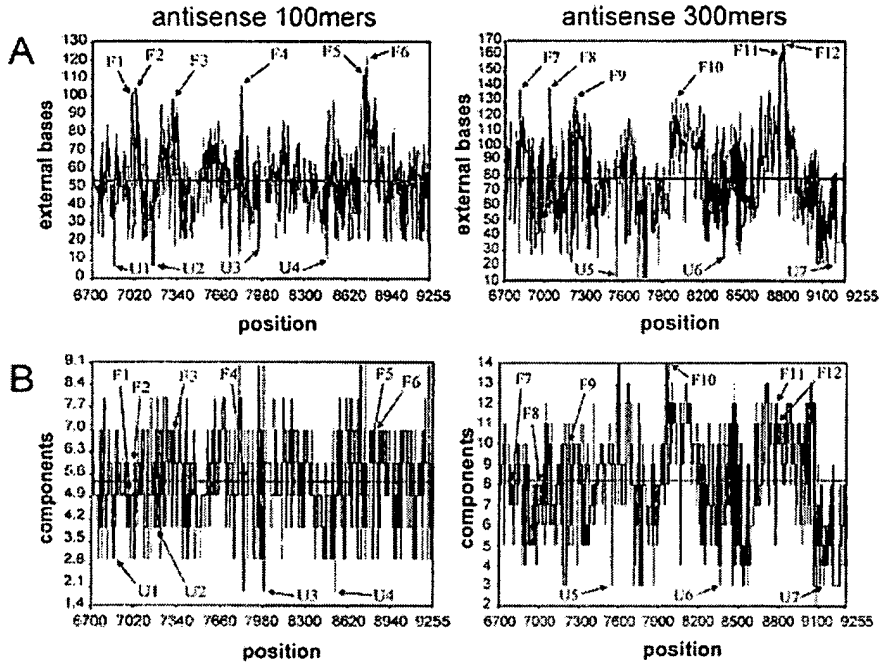

Figure 1. Distributions and variances of selection parameters of endogenously transcribed antisense RNA 100mers and 300mers along the HIV-1 *gag* gene. Calculated numbers of external bases (**A**) and components (**B**) are plotted versus the reverse complement (first position of the analyzed antisense window) of the HIV-1 primary transcript. For example, position 9255 corresponds to position 1 of the HIV-1 primary transcript or to position 455 of HIV-1 clone pNL4-3 (EMBL accession no. REHIVNL4), respectively. Parameters corresponding to selected constructs are indicated. Mean values are indicated by red lines.

gene are shown in Figure 1. The two sets of antisense genes were selected according to the selection scheme described above. First, a set of 10 antisense genes resulting in a RNA transcript of 310 nt total length containing a central antisense portion of 100 nt (antisense 100mers; Fig. 2A). Six of these structures (F1–F6) were regarded as favorable structures in terms of effectiveness according to the selection parameters (Figs 1 and 3A) and four structures (U1–U4) were predicted to be unfavorable. Secondly, a set of nine antisense genes resulting in RNA transcripts of 510 nt total length containing a central antisense portion of 300 nt (antisense 300mers) was selected (Fig. 2B), comprising favorable structures F7–F12 and unfavorable structures (U5–U7). As shown in Figure 3A, the favorable structures contain 10-fold (7-fold) higher numbers of external bases and 2-fold (3-fold) higher numbers of structural components in the case of the antisense 100mers (300mers).

Intracellular steady-state levels of HIV-1 *gag*-directed endogenous antisense RNA transcripts

Steady-state levels of endogenously transcribed antisense RNA were measured 24 h after transfection of the corresponding antisense genes into 293T cells by quantitative RT–PCR. All steady-state levels of antisense RNA (Fig. 4A) were standardized to the corresponding levels of *GAPDH* mRNA and were compared to the level of RNA transcribed from the insert-free vector backbone. The average values indicate ~2-fold lower steady-state levels of the favorable structures compared to unfavorable structures (Fig. 4A). Individual transcripts however, such as F7 and F8, showed up to 10-fold lower steady-state levels compared to the average of all favorable antisense 300mers. Interestingly, higher levels were observed in the case of the shorter antisense transcripts.

Efficacy of HIV-1 *gag*-directed antisense genes in 293T cells

To measure antisense RNA-mediated inhibition in mammalian cells, we used a transient co-transfection assay for HIV-1 replication which represents a valid system: human 293T cells were co-transfected with a mixture of antisense RNA expression plasmid and the cloned infectious proviral HIV-1 DNA pNL4-3 (EMBL accession no. REHIVNL4) by the calcium phosphate co-precipitation protocol (9). Virus was released into the cell culture supernatant and quantified by HIV-1 p24 antigene ELISA. The ratio of both types of DNA was chosen such that levels of inhibition were in a range that allowed comparison of all constructs. All constructs harboring a 100 nt antisense stretch showed significant inhibition, though no significant difference was observed between the group of favorably predicted antisense genes and those predicted to be less effective (Fig. 3B). For the constructs containing a 300 nt stretch of antisense sequences, a 2-fold difference in efficacy was measured among the two groups of theoretically selected antisense genes (Fig. 3B), which is statistically significant. All positively selected species showed stronger inhibition of HIV-1 compared to all negatively selected structures. The probability of observing this relation on a statistical basis is 0.012. It is

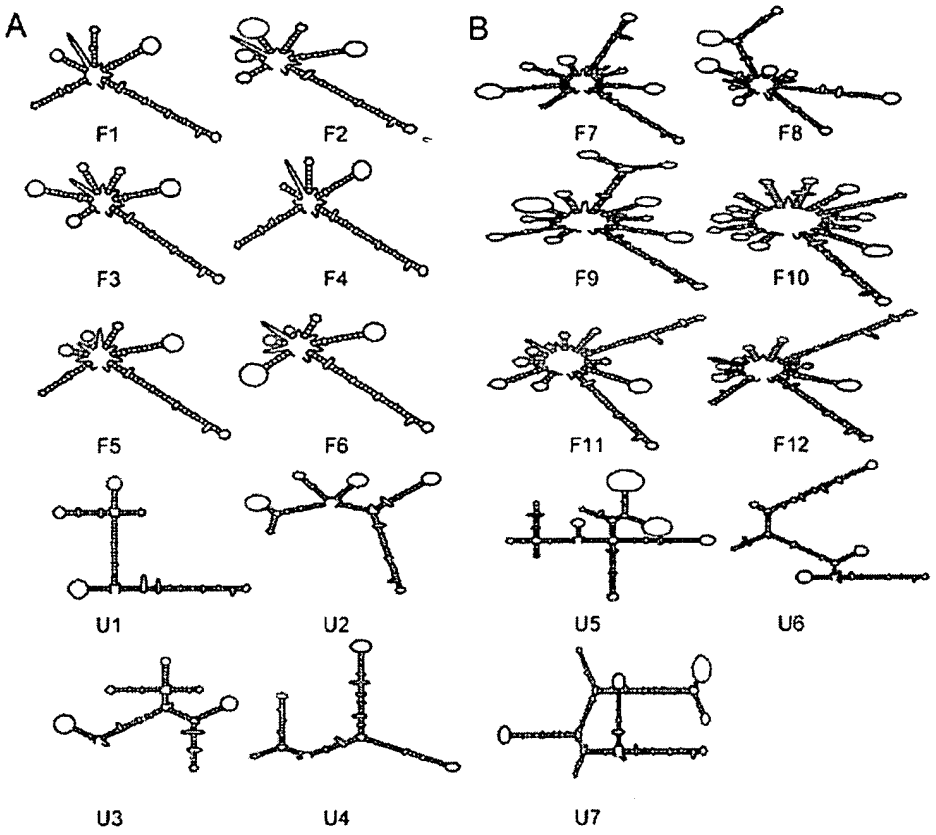

Figure 2. Computer-predicted minimum free energy secondary structures of HIV-1 *gag*-directed antisense transcripts with (**A**) a 100 or (**B**) a 300 nt antisense sequence portion. Antisense sequence stretches of favorable structures (F1–F12) are colored green; antisense sequence stretches of unfavorable structures (U1–U7) are colored red; black represents non-complementary vector-derived sequences identical for all RNA transcripts. Structural elements relevant for the computational selection are specified in structure F1. Components are shaded; external bases are not shaded.

reasonable to assume that intracellular concentrations of antisense RNA are important for efficacy. Thus, we defined an antisense effect of the different antisense genes by considering the measured intracellular RNA steady-state levels in order to compare the antiviral efficacy. As shown in Figure 4B, the dynamic range between individual species as well as between positively and negatively selected antisense structures became significantly greater. On average, the positively selected antisense structures showed an 11-fold higher antisense effect compared to the negatively selected species in the case of the antisense 300mers, and even for the antisense 100mers, for which no difference was observed in virus replication, a 2- to 3-fold higher antisense effect was calculated (Fig. 4B).

Relationship between secondary structures of HIV-1 *gag*-directed antisense transcripts and efficacy

The results presented here indicate that, with some limitations, the previously identified selection parameters (3) can be used for improved design of endogenous HIV-1-directed antisense transcripts or antisense genes. Structures with higher numbers of external bases and components are stronger inhibitors of HIV-1 replication in the case of a 300 nt but not a 100 nt antisense stretch. However, the correlation between the theoretically calculated structural parameters of antisense RNA and their efficacy in living cells is of a qualitative and not a quantitative nature.

DISCUSSION

The theoretical approach presented here seems to be suitable to significantly improve the design of antisense genes directed against HIV-1. In the case of the constructs with a 300 nt antisense portion all favorably predicted structures (F7–F12) showed improved inhibition of HIV-1 replication. Conversely, without consideration of intracellular RNA steady-state levels, no influence of computational selection on viral replication was observed in the case of structures with a 100 nt antisense portion. The average inhibitory potential of the antisense 100mers was found to be between the efficacy of positively and negatively selected antisense 300mers. The failure of the computational prediction in the case of the shorter antisense stretches could be attributed to a structure-dominating effect of

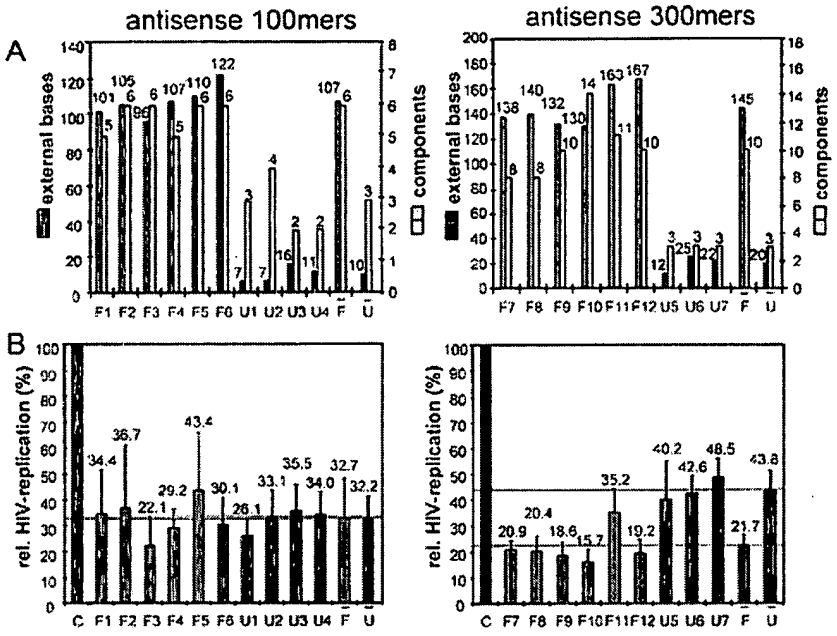

Figure 3. Relationship between predictable RNA structure parameters, the numbers of external bases and components (**A**), and the inhibition of HIV-1 replication in 293T cells by theoretically selected antisense genes (**B**). Predicted structures were selected for high numbers of external bases and high numbers of components. Values for HIV-1 replication are mean values of 5 × 3 co-transfection experiments. The endogenously transcribed antisense RNAs are 310 or 510 nt in length [without a poly(A) tail] and contain a 100 or 300 nt antisense portion respectively. Green bars (solid or hatched) represent favorable structures (F1–F12) and red bars (solid or hatched) unfavorable structures (U1–U7); black bars represent the effect of the cloning vector itself (100% HIV-1 replication). Letters with bars indicate the average values of favorable or unfavorable RNA structures. Mean values are additionally indicated by horizontal lines.

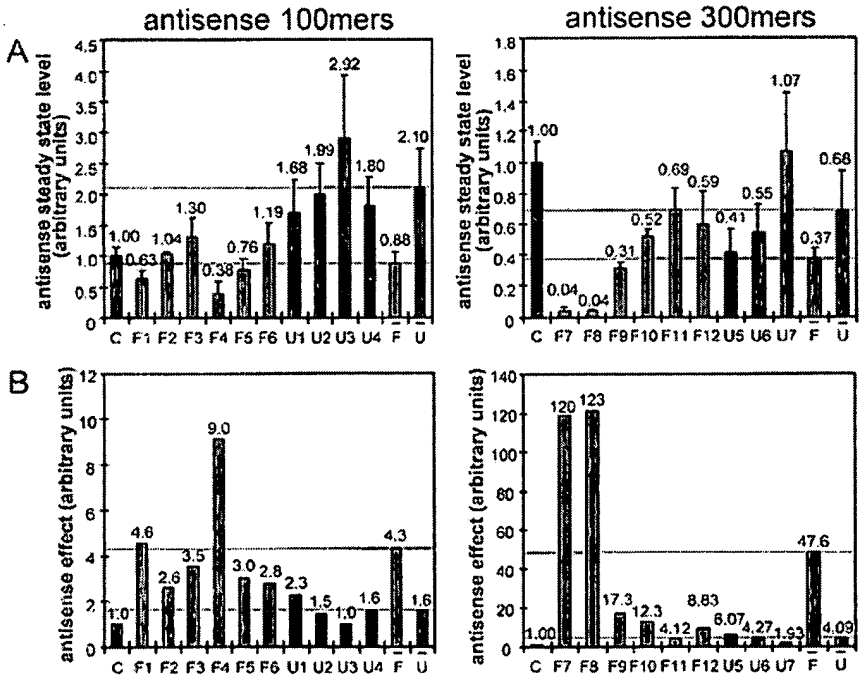

Figure 4. (**A**) Steady-state levels of HIV-1 *gag*-directed antisense RNA species in 293T cells determined by quantitative RT–PCR. Values are standardized to levels of *GAPDH*. Values are mean values of three experiments. (**B**) Antisense effects of selected antisense genes according to the equation: antisense effect = 100/[(HIV-1 replication [p24]) × (steady-state level)]. For the color coding see the legend to Figure 3. The values obtained for the cloning vector (lane C) are set to one.

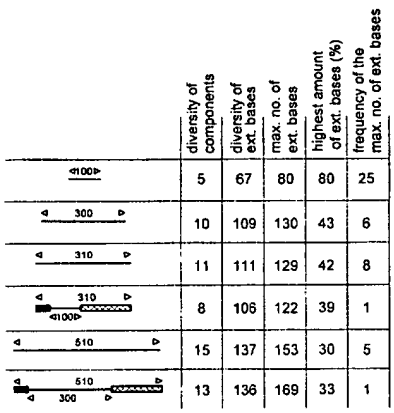

Figure 5. Characterization of hypothetical HIV-1 *gag*-directed antisense RNA structure spaces for the schematically depicted sequence constructs of different chain length. Variable antisense portions are represented by single lanes, black boxes represent transcribed nucleotides of the CMV promotor and hatched boxes represent the SV40 poly(A) tail. A set of theoretically determined parameters was calculated with the program Space. ext., external; max., maximum; no., number. Diversity of components and diversity of ext. bases reflect the dynamic range of these parameters within the analyzed sequence space. Max. no. of ext. bases reflects the highest number of external bases observed for a single species. Highest amount of ext. bases indicates the percentage of external bases within the species with the maximum number of external bases. Frequency of the max. no. of ext. bases indicates the number of structures with the maximum number of external bases found during each analysis.

the relatively long constant non-complementary vector-derived sequences at the termini of these molecules. This idea is supported by the observation that the vector-derived portions fold into identical structural sub-domains within many positively and negatively selected species (Fig. 2A). For example, an identical stem–loop is predicted for the CMV promoter-derived 5′-terminal sequences within structures F6, U1 and U2. Another extended stem–loop structure is predicted within the SV40 poly(A)-derived sequence portion of structures F1–F6, U1 and U2 (Fig. 2A). The ratio of non-complementary to complementary bases is 210/100 = 2.1 in the case of the antisense 100mers (310 nt in length) and 210/300 = 0.7 in the case of the antisense 300mers (510 nt in length). These constant vector-derived terminal sequences significantly reduced the diversity of structures concerning the two selection parameters, the numbers of external bases and components compared to completely complementary sequences of identical chain length. This becomes obvious when regarding the variability of the two selection parameters of different HIV-1 *gag*-directed antisense structure spaces (Fig. 5). As expected, the structural diversity, with respect to external bases and components, was significantly higher in the case of antisense 510mers compared to antisense 310mers (fully complementary sequences each), which is due to the higher potential of alternative base pairing within the longer sequences. However, when substituting 33 nt located at the 5′-ends and 177 nt located at the 3′-ends by invariable vector sequences, this diversity was reduced more dramatically in the case of the 310mers (Fig. 5). Further, the frequency of structures with the maximum number of external bases was much higher in the case of completely complementary sequences and was more strongly reduced by terminal vector-derived sequences in the case of 310mers compared to 510mers (Fig. 5). These considerations are compatible with the previous observation that the computational design of HIV-1 *gag*-directed antisense RNA of 100 nt in length can dramatically enhance inhibition of HIV-1 replication if the RNAs are transcribed *in vitro* with a maximum of six non-complementary bases at the 5′-end prior to transfection of mammalian cells (3). Terminal vector-derived sequences avoid complementary external bases to be located in terminal positions. Especially, complementary terminal external bases were found to be critical for annealing and antiviral activity of HIV-1-directed antisense RNA.

On a statistical basis long antisense RNA molecules anneal faster with the target strand than shorter ones and, thus, are potentially stronger inhibitors (10). It is reasonable to assume that, analogous to *in vitro* or *in vivo* selection strategies, the success of computational selection is also correlated with the structural diversity of the initial pool. However, an arbitrary elongation of the antisense stretch up to the length of the target sequence would reduce the sequence and structure diversity to one. This would result in a statistically potent but probably sub-optimal molecule. The probability of identifying favorable structures in terms of annealing and efficacy decreases with the length of the antisense molecule (Fig. 5). When regarding the fully complementary sequences, the highest number of external bases among all HIV-1 *gag*-directed antisense species was 80% in the case of 100mers, 43% in the case of 300mers, 42% for 310mers and only 30% for 500mers. Similarly, in the case of endogenous transcripts, the highest number of external bases was 39% for 310mers and 33% for 510mers. The longer the antisense molecules were, the closer to the average they became in terms of structural features. Thus, in order to improve the computational design of endogenously transcribed antisense RNA and the efficacy of selected species it seems to be promising to reduce the ratio of non-complementary to complementary sequences, particularly by minimizing the non-complementary sequence portions rather than by elongating the antisense stretch as one might expect when extrapolating the results of the analyzed antisense 100mers and 300mers. On the basis of these interpretations it seems to be attractive to use the polymerase III promotor, which generates a minimum of transcribed promotor sequences, for the future design of antisense genes.

Despite a potentially stronger influence of vector-derived sequences in the case of the antisense 100mers, the question has to be addressed of why the computational selection does not improve the design of antisense genes in terms of efficacy, although from the theoretical point of view the secondary structure prediction should be more reliable in the case of shorter sequences. One explanation could have been a thermodynamically more stable local target region in the case of the positively selected HIV-1-directed antisense 100mers. A strong influence on annealing and inhibition of gene expression of this, so-called, local folding potential of the target sequence has been investigated previously (4). However, in the case of the HIV-1-directed antisense 100mers there is no hint that local thermodynamic target stability might be responsible for the lack of correlation between RNA secondary structure prediction and efficacy in human cells (Table 1). From a statistical

Table 1. ΔG values of intramolecular structure formation of local targets and antisense sequences

Antisense RNA	Target position[a]	ΔG target (kcal/mol)	Average ΔG targets (kcal/mol)	ΔG antisense species (kcal/mol)	Average ΔG antisense species (kcal/mol)
HIV 100					
F1	2617–2716	–5.5	–9.4	–36.1	–37.5
F2	2587–2686	–10.3		–43.2	
F3	2302–2401	–8.8		–37.4	
F4	1768–1867	–15.4		–43.5	
F5	814–913	–6.9		–36.5	
F6	785–884	–9.2		–28.5	
U1	2748–2847	–12.6	–11.9	–40.2	–41.4
U2	2457–2556	–10.8		–45.4	
U3	1635–1734	–12.3		–38.5	
U4	1094–1193	–12.0		–41.4	
HIV 300					
F7	2601–2900	–35.3	–47.6	–63.0	–68.9
F8	2374–2673	–44.7		–65.5	
F9	2173–2472	–51.0		–78.9	
F10	1440–1739	–50.0		–77.1	
F11	628–927	–51.7		–63.4	
F12	607–906	–53.0		–65.2	
U5	1699–1998	–48.0	–58.0	–68.5	–80.3
U6	1047–1346	–44.3		–72.7	
U7	275–574	–81.8		–99.8	

[a]Sequence published as EMBL accession no. REHIVNL4.

point of view, thermodynamics should rather support duplex formation between the target RNA and favorably structured antisense molecules. A more trivial explanation would assume that other biochemical parameters than annealing, such as intracellular stability (11), subcellular localization (12) or intracellular transcription rate, are important for the efficacy of the *gag*-directed antisense 100mers. The sum of all these anabolic and catabolic processes is reflected in the intracellular RNA steady-state levels determined in the absence of target RNA, which are significantly lower in the case of the favorable antisense structures (Fig. 4A). This is compatible with the previous observation that such selected potentially fast annealing structures are less stable in cell extracts (3). Low intracellular RNA stability would necessarily result in low RNA steady-state levels. During the infectious assay HIV-1 transcripts were present and annealing with the target sequence could compete with RNA degradation, probably resulting in RNA levels lower than those determined in the absence of target sequences. While fast annealing is related to higher efficiency of antisense RNA, lower RNase resistance might just have equalized this effect in the case of the favorable antisense 100mers but not the 300mers. The annealing rate constants of the individual species are not known. The 3-fold lower RNA steady-state levels of the antisense 300mers might be a hint of an endonucleolytic rather than an exonucleolytic degradation pathway of endogenously transcribed artificial RNA.

There are controversal opinions about the usefulness of single secondary structure predictions for the evaluation of favorable antisense sequences in the antisense field. This and previous work (3,6) indicate that a more extensive computational secondary structure analysis can significantly support the design of antisense compounds. We present a first approach to improve the design of antisense genes compared with a purely statistical basis. We suggest that when using a low ratio of vector-derived to complementary sequence portions, the computer-aided design of endogenously expressed antisense RNA significantly improves the efficacy of antisense genes. The potential of computational selection is more dramatically reflected in the definition of an antisense effect which considers the lower intracellular steady-state levels and, hence, lower nuclease resistance of favorable antisense RNA molecules. Thus, it seems promising to consider RNA-stabilizing structural domains during computational selection to design fast annealing and more stable antisense molecules. Similarly, RNA transport signals (13,14) considered during the selection protocol might help to direct endogenous antisense transcripts to subcellular locations where they encounter their target. The method presented here is time- and labor-saving and, hence, may be applied prior to any kind of experimental antisense gene design.

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REFERENCES

1. Wagner,E.G.H. and Simons,R.W. (1994) *Annu. Rev. Microbiol.*, **48**, 713–742.
2. Sczakiel,G. (1997) *Antisense Nucleic Acid Drug Dev.*, **7**, 439–444.
3. Patzel,V. and Sczakiel,G. (1998) *Nature Biotechnol.*, **16**, 64–68.
4. Sczakiel,G., Homann,M. and Rittner,K. (1993) *Antisense Res. Dev.*, **3**, 45–52.
5. Rittner,K. and Sczakiel,G. (1991) *Nucleic Acids Res.*, **19**, 1421–1426.
6. Patzel,V., Steidl,U., Kronenwett,R., Haas,R. and Sczakiel,G. (1999) *Nucleic Acids Res.*, **27**, 4328–4334.
7. Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
8. Senger,M., Glatting,K.-H., Ritter,O. and Suhai,S. (1995) *Comput. Methods Programs Biomed.*, **46**, 131–141.
9. Chen,C. and Okayama,H. (1987) *Mol. Cell. Biol.*, **7**, 2745–2752.
10. Patzel,V. and Sczakiel,G. (1999) *J. Mol. Biol.*, **294**, 1127–1134.
11. Case,C.C., Roels,S.M., Jensen,P.D., Lee,J., Kleckner,N. and Simons,R.W. (1989) *EMBO J.*, **8**, 4297–4305.
12. Hormes,R., Homann,M., Oelze,I., Marschall,P., Tabler,M., Eckstein,F. and Sczakiel,G. (1997) *Nucleic Acids Res.*, **25**, 759–775.
13. Grimm,C., Lund,E. and Dahlberg,J.E. (1997) *EMBO J.*, **16**, 739–806.
14. Nigg,E.A., Baeuerle,P.A. and Lührmann,R. (1991) *Cell*, **66**, 15–22.

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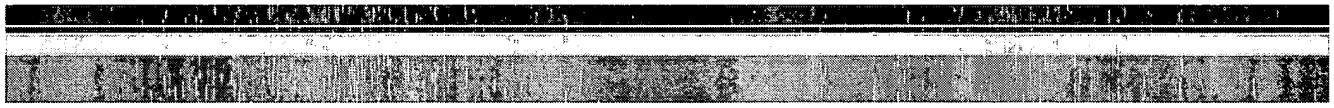

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